Vol 8, Issue 1, February 2004

ELSEVIER

Functional protein microarrays: ripe for discovery

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The manufacture and use of protein microarrays with correctly folded and functional content presents significant challenges. Despite this, the feasibility and utility of such undertakings are now clear, and exciting progress has recently been demonstrated in the areas of content generation, printing strategies and protein immobilization. More importantly, we are now beginning to enjoy the fruits of these efforts as functional protein microarrays are being increasingly employed for biological discovery purposes. Recent examples of this include the characterization of autoantibody responses, antibody specificity profiling, protein–protein domain interaction profiling and a comprehensive characterization of coiled-coil interactions. The best, however, is yet to come.

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Current Opinion in Chemical Biology 2004, 8:8–13

This review comes from a themed issue on
Proteomics and genomics
Edited by Michael Snyder and John Yates III

1367-5931/$ – see front matter

DOI 10.1016/j.cbpa.2003.12.005

Introduction

The human genome project has catalyzed the development of new large-scale approaches to addressing biological questions. A prime example of this is the now common use of DNA microarrays for large-scale mRNA expression analysis. Functional protein arrays (microarrays with immobilized functional proteins, Figure 1) are a logical extension of DNA microarrays. However, the manufacture and use these microarrays presents significant challenges compared with their DNA counterparts. In fact, before the first report [1••], many doubted it could even be done at all!

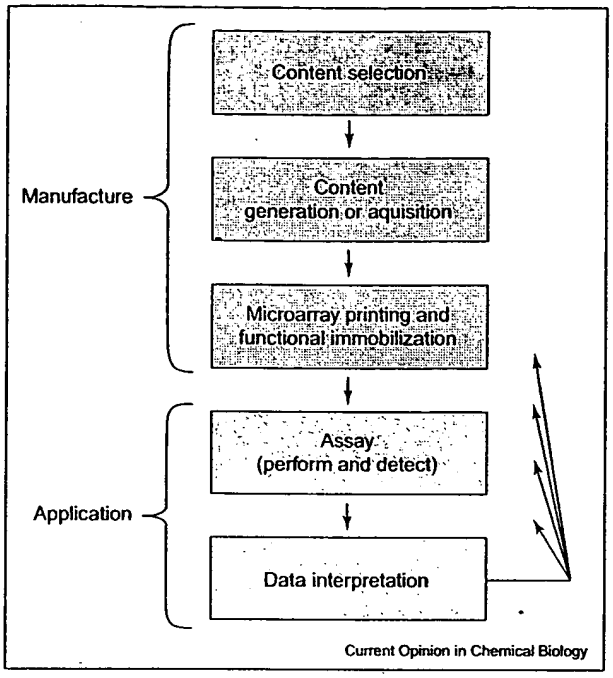
While tractable, the manufacture of protein microarrays is not for the faint of heart. Considerable challenges still exist in terms of content generation, printing, functional immobilizing, and detection. The current state and recent advances in each of these areas is briefly reviewed here. The end goal of all of this research, however, is its application towards developing meaningful insights and discovery in biology. These applications span a wide range, from molecular interactions for protein functional characterization to optimization of drug–protein interactions, from profiling of enzyme substrates to profiling enzymatic activities (Table 1). Functional protein microarrays clearly have the potential to make significant contributions to both basic and applied research [2]. Although many of these applications remain at the proof-of-concept stage, there have recently been important advances in reducing concept to practice, which I review here.

Functional content

Perhaps the most significant barrier to manufacture of functional protein microarrays is generation of the protein content itself. Early efforts in creating protein expression collections focused on more random approaches, such as cDNA libraries cloned into *Escherichia coli* expression vectors [3]. In fact, the use of such collections in protein microarrays has recently been described [4]. However, a significant drawback of such approaches is the relatively low yield of folded full-length proteins, even in the presence of positive selection. More focused efforts to express a random set of human proteins in *E. coli* have given reasonable yields of ~60%; ~80% of which could be purified under non-denaturing conditions [5]. Unfortunately, proteins expressed in *E. coli* lack the post-translational modifications observed in eukaryotic proteins, which can be required for proper protein function. Insect cells provide an example of a eukaryotic expression system successfully adapted to high-throughput protein expression [6]. In recent experiments, approximately 15% of human proteins expressed in insect cells showed detectable levels of phosphorylation (Figure 2). High-throughput expression of human proteins in COS cells (a cell line established from monkey kidney cells) has also been reported [7]. In practice, different proteins often require different expression hosts and vector constructs for optimal expression. Given this need, an ability to readily shuttle inserts from one vector to another will be important. Commercial systems such as Invitrogen's Gateway and BD Clontech's Creator systems meet this requirement, although licensing restrictions with commercial systems can be problematic. Expression clone sets such as the human FLEXgene repository [8,9], which is primarily generated from the Mammalian Gene Collection [10], and the *Caenorhabditis elegans* ORFeome collection [11••] were generated in such systems.

An alternative to expression clones, cell-free expression, is not reviewed here, but has apparently been adapted to 96-well format with good success [12], and therefore shows promise for the highly parallel protein expression capabilities required to generate protein microarrays. Regardless of the mode of expression, however, there is a subsequent need to purify large numbers of different proteins in parallel with reasonable yields. While a

Figure 1



Manufacture and application of functional protein microarrays. This high-level flowchart summarizes the major steps required in the manufacture and use of functional protein microarrays.

96-well plate format makes this easy to do in principal [5–7], doing it well is a significant, albeit surmountable, engineering challenge.

Microarray printing

There are two general approaches to microarray printing: contact and non-contact. Given the requirement to array large numbers of different proteins, contact printing is currently the most suitable choice, although non-contact printing of functional protein microarrays is certainly possible [13].

Recent advances in microarray printing include a laser transfer technique [14], microfabricated fountain pens for high-density array construction [15], as well as a novel affinity contact printing procedure employing a multi-use stamp [16]. Cooks' group at Purdue University recently described an exciting proof-of-concept using electrospray ionization of a protein mixture followed by mass ion separation and sequential soft landing deposition onto a surface to create a protein array [17••]. This technology, while promising, has many challenges ahead, including improving print speed as well as addressing protein quantity, identity and functionality.

Functional immobilization

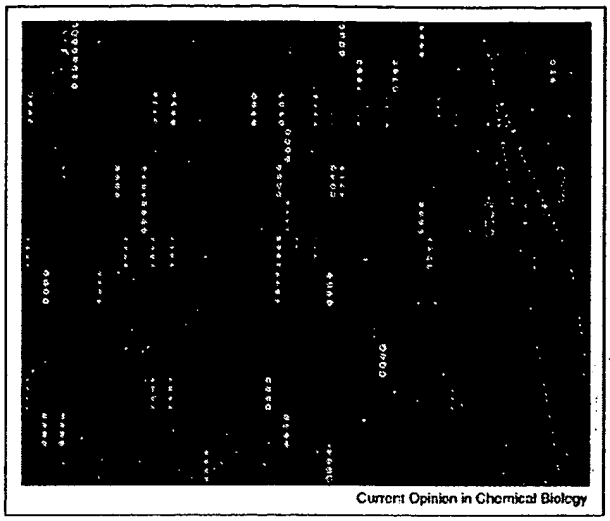
Basic strategies for protein immobilization consider covalent versus noncovalent and oriented versus random

Table 1

Applications of functional protein microarrays. A summary of many of the basic and drug research applications of functional microarray experiments.

Experiment	Basic research application	Drug research application
Protein–protein interaction profiling	Pathway mapping Protein interaction mapping Protein function determination K_D estimation	Target discovery Early target validation
Protein–lipid interaction profiling	Pathway mapping Protein function determination	Target discovery Early target validation
Protein–DNA interaction profiling	Pathway mapping DNA-binding protein discovery	Target discovery Early target validation
Protein–small-molecule interaction profiling	Pathway mapping Metabolomics	Drug rescue Target/mechanism determination Alternate target identification Specificity profiling IC_{50} determination Lead optimization
Substrate assays	Pathway mapping Substrate identification	Target discovery Early target validation
Enzymatic activity profiling	Enzyme activity discovery	Target discovery Early target validation
Enzymatic activity assay	Enzyme kinetics	Specificity profiling IC_{50} determination Lead optimization

Figure 2



Phosphorylation of human proteins expressed in insect cells. Approximately 1000 different human proteins expressed in insect cells were spotted onto a microarray in quadruplicate. Phosphorylation was detected by the phosphate binding dye Pro-Q™ (Molecular Probes). Approximately 15% of proteins showed detectable signal. A portion of the image is shown.

attachment, as well as the nature of the surface itself. Some examples of oriented attachment include immobilization of His-tagged proteins on nickel slides [1••] and of biotinylated proteins to avidin-coated slides [18•]. There is evidence that, for some proteins, oriented attachment enhances the fraction of available and/or active protein [19]. However, several demonstrations of functional proteins using random attachment have now been published [20,21], including most of those in the 'New applications' section [1••,4,22,23,24•,25••,26•,27••,28]. Similarly, successful use of microarrays generated by both covalent and noncovalent attachment has been reported. Also, a large number of surfaces have been demonstrated to be compatible with functional protein microarrays, from acrylamide- and nitrocellulose-coated to aldehyde and poly-L-lysine modified glass slides. Self-assembled monolayers provide yet another technique for the development of biocompatible surfaces [29,30]. G-protein-coupled receptors have even been functionally microarrayed onto γ-aminopropylsilane slides [31–33]. Recent developments in lipid immobilization may provide improved capabilities in membrane protein immobilization [34–36].

An ideal surface or immobilization for all proteins and all applications doesn't exist, so work to develop biocompatible surfaces is both ongoing [33] and important. However, as is demonstrated in the literature, it is clear that a large number of proteins retain functionality over a wide range of surface and immobilization conditions. Thus, despite the lack of an ideal universal surface or immobilization approach, existing methods are more than adequate for many applications.

Detection

Most applications of functional proteome microarrays for interaction or substrate detection have employed some type of labeling strategy; usually fluorescent [1••,4,23,24•,25••,26•,28,37,38], colorimetric [22] or radioactive [23,39]. One noteworthy development in fluorescent protein labeling is the puromycin-based labeling strategy [40,41], which enables fluorescent labeling simultaneously with cell-free expression. Although label-free detection technologies, such as surface plasmon resonance [42], mass spectrometry [43] and others, are highly desirable, their availability and sensitivity have not been high enough to have come into common use for functional protein microarrays. An interesting development in label-free detection is an alamethicin-based detection strategy that measures the blocking of channel current through a planar lipid bilayer upon binding [44]. Regardless of the physics employed, the development of practical, robust and sensitive label-free detection strategies will be tremendously valuable.

Recent applications

The early literature in the functional protein microarray field consists primarily of proof-of-concept work. The pioneering paper of MacBeath and Schreiber, for instance, demonstrated three important proofs for protein microarrays; protein–protein binding, protein kinase substrate phosphorylation and small-molecule–protein binding [23]. Around the same time, modified polyacrylamide gel pads were demonstrated for protein immobilization and subsequent immunoassay and enzymatic kinetic measurements (horseradish peroxidase, alkaline phosphatase, β-D-glucuronidase in the presence or absence of inhibitors) [22]. More recent work from Yao's laboratory has demonstrated the novel application of mechanism-based inhibitors for activity-based detection of enzymes using protein microarrays [37].

The first reported use of functional protein microarrays for biological discovery purposes was reported by Snyder's laboratory, which manufactured and used yeast proteome microarrays for protein interaction and lipid binding screens [1••]. Since that time, a slowly increasing number of discovery-based papers have been published.

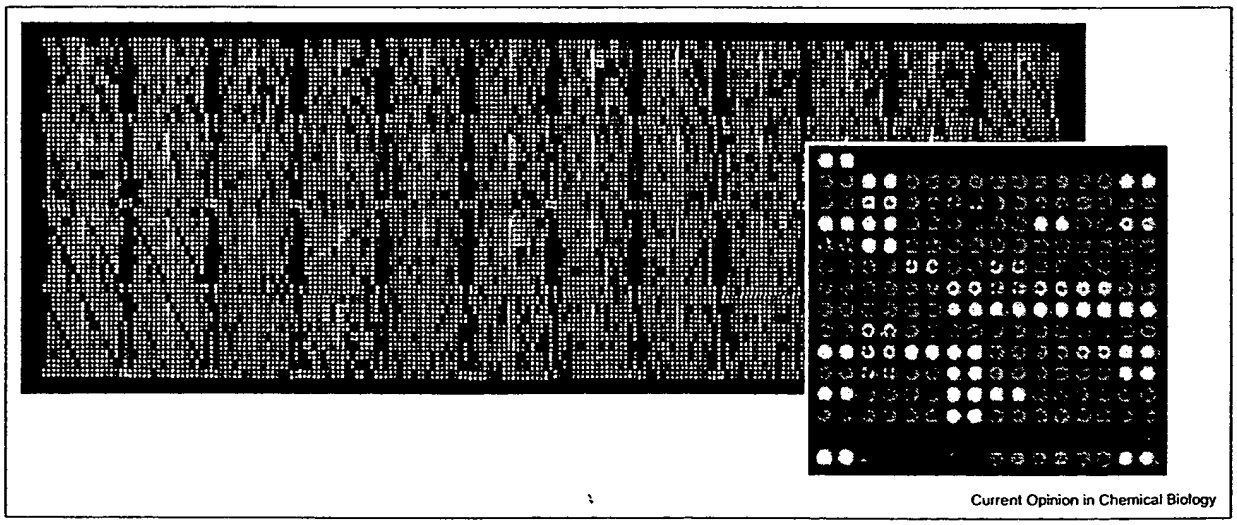
In 2002, Espejo *et al.* [24•] reported the use of protein domain microarrays to identify novel protein–protein interactions. In this study, peptide motifs were used to demonstrate proper binding specificity of several glutathione S-transferase fusions of protein interaction modules, such as WW, SH (Src homology), forkhead-associated, plekstrin homology and FF domains. The arrays were generated by microarraying proteins at 1 mg/ml onto

nitrocellulose-coated glass slides, and were probed with biotinylated peptides prebound to fluorescently labeled streptavidin. The same type of slides were also probed with total-cell lysates, re-probed with an antibody raised against either Sam68 or SmB′, and detected with fluorescently labeled secondary antibody. Whereas Sam68 displayed the expected binding partners, SmB′ showed an unexpected degree of binding to SH3 domains, leading the authors to hypothesize a role for SmB′ as a spliceosome attachment scaffold.

More recently, Newman and Keating [25••] used protein arrays to test all binary interactions of 49 (out of 55 known) coiled coil strands from human basic-region leucine zipper transcription factors. After high-performance liquid chromatography purification, the peptides were arrayed in quadruplicate onto aldehyde-derivatized glass slides. To reduce homodimerization, reduced and guanidine hydrochloride denatured peptides were printed and probed with denatured fluorescent peptides, which were rapidly diluted immediately before use. Both strong (~50 nM) and weak (~3 μM) binding could be detected, and high specificity was observed in the experiments, with only ~14% of all measured pairs showing interactions, and only ~6% showing strong interactions. The array results agreed well with follow-up circular dichroism studies. As expected, peptides within families tended to show similar interactions, whereas peptides from different families had distinct interactions. In addition, several previously undetected interactions were reported, permitting the generation of some exciting biological hypotheses. It will be interesting to watch these and other hypotheses be tested in the near future.

A proof-of-concept experiment for antibody specificity profiling was recently published by Lueking *et al.* [4]. Polyacrylamide-coated glass slides were used to create microarrays of ~2400 human fetal brain cDNA expression clones that express protein in *E. coli*. These microarrays were used to probe with mouse monoclonal α-GAPDH (glyceraldehydes phosphate dehydrogenase) and α-HSP90β (heat-shock protein) antibodies. Both antibodies preferentially recognized their cognate antigens, but additional unrelated cross-reactive proteins were also identified. In a similar study employing 96 *Arabidopsis* proteins, only specific antigen binding was observed [38]. It is likely, however, that most of the proteins used in these studies were unfolded. An example of antibody specificity profiling with whole-proteome microarrays using folded proteins was reported by Michaud *et al.* [45•]. In this case, both polyclonal and monoclonal antibodies generated towards yeast proteins were probed against a yeast proteome microarray. Not surprisingly, monoclonal antibodies tended to show more specificity than polyclonal antibodies. However, even monoclonal antibodies exhibited demonstrable cross-reactivity. Interestingly, most cross-reactivity could not be predicted *a priori* on the basis of sequence analysis, suggesting that empirical approaches to profiling antibody specificity should be an important consideration when developing or using antibodies for research or medical purposes. This new approach to profiling antibody specificity will

Figure 3



Fluorescent Image of a Yeast ProtoArray™. Microscope slides were spotted with >4000 different proteins cloned from and expressed in yeast. Proteins were detected using a Cy5-labeled antibody directed against an epitope tag. Slides were scanned using an Axon 4000B microarray laser scanner. The scan of an entire 48-subarray slide is shown at the top of the figure, while an image of just one subarray is shown in the bottom. Copyright 2003 Protometrix Inc. Reprinted with permission.

become increasingly powerful as larger sets of proteins become available.

Robinson *et al.* [26•] reported the use of autoantigen arrays for screening human disease sera. Microarrays were constructed using 196 autoantigens (including proteins, protein complexes, peptides and DNA) corresponding to eight different autoimmune diseases. These autoantigens were spotted onto 1152 features on poly-L-lysine-coated slides and probed with fluorescently labeled human serum. Using this approach, the authors observed distinct autoantibody profiles consistent with each disease. In addition, they demonstrated the ability to use these microarrays for epitope mapping of autoantibody response. In follow-up work, 'myelin proteome' microarrays were used to characterize autoantibody epitope spreading in experimental autoimmune encephalomyelitis, a mouse model for multiple sclerosis [28]. Microarray analysis was subsequently used to guide the development of tolerizing vaccines. This work has clearly demonstrated the potential of autoantigen arrays in characterizing and ultimately treating autoimmune disease. The approach will ultimately be supplemented by the identification of currently unknown autoimmune antigens. As an early example of this, Lueking *et al.* recently reported screening low complexity human protein arrays against autoimmune sera [4], identifying some interesting leads for further analysis. Finally, a 430-peptide/protein simian-human immunodeficiency virus microarray was recently used to profile macaque immune response to vaccination, with results predictive of survival [27••]. In addition, three novel viral epitopes were identified.

Conclusions

Functional protein microarrays hold enormous potential for biological discovery and drug development. Significant attention has been and will continue to be devoted to technology development. However, several recent literature publications highlight the fact that, despite continuing technological challenges, the current state of the art is such that this huge potential is now being unleashed. The imminent commercial introduction of functional protein microarrays [46] (Figure 3) will only accelerate this process.

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- • of special interest
- •• of outstanding interest

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